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IN VITRO STUDIES ON THE EFFECTS OF CELL-FREE COELOMIC FLUID, CALCIUM, AND/OR MAGNESIUM ON CLUMPING OF COELOMOCYTES OF THE SEA STAR *ASTERIAS FORBESI* (ECHINODERMATA: ASTEROIDEA)

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Abstract

In asteroid echinoderms the loss of coelomic fluid due to injury is prevented by the clumping of coelomocytes at the site of the wound. Plasma (cell-free coelomic fluid = CF) coagulation has not yet been demonstrated in these animals. An *in vitro* system was used to quantify the effects of CF, Ca^{2+}, and/or Mg^{2+} on coelomocyte clumping in the sea star *Asterias forbesi*.

The results show that the coelomocytes of *A. forbesi* require threshold levels of Ca^{2+} and/or Mg^{2+} for clumping *in vitro*, and these levels depend on whether the ions are used separately, in combination, or as components of CF. The findings also suggest that the *in vitro* coelomocyte clumping is mediated by a factor present in CF which requires Ca^{2+} and Mg^{2+} to be effective. A two-phase clumping, consisting of a fast phase followed by a slow phase, is also demonstrated.

The observed biphasic clumping is explained by the existence of two functional subpopulations among the coelomocytes which differ in their permeability characteristics and ability to establish surface adhesiveness for clumping. Morphological identities of these two subpopulations remain to be ascertained.

Introduction

While it is known that the clumping of the coelomocytes occurs as a means of hemostasis in asteroid echinoderms (sea stars) (see reviews by Endean, 1966; Needham, 1970; Belamarich, 1976; Kanungo, 1982), controversy exists as to the type of coelomocyte and the mechanism involved in such cellular clumping. (In this paper the terms "aggregation" and "agglutination" are used interchangeably with "clumping" of coelomocytes *in vivo* or *in vitro*, and the term "cell" is used to refer to the "coelomocyte.")

Boolootian and Giese (1958, 1959) maintained that bladder amoebocytes transformed into filiform amoebocytes which then agglutinated to form plasmodial clots in eight species of sea stars which they investigated. The filiform stage was, therefore, viewed as a precoagulant phase. Johnson and Beeson (1966) on the other hand, reported that the filiform stage was not required to initiate or to maintain coelomocyte clumps in the sea star *Patiria miniata*.

In analyzing the mechanism of of coelomocyte agglutination, Boolootian and Giese (1959) also observed that the agglutination was not dependent on calcium but on the formation of disulfide linkages. However, Jangoux and Vanden Bossche

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Abbreviations: CF, cell-free coelomic fluid (plasma); CMFSS, calcium- and magnesium-free salt solution; Hepes, N-2-hydroxyethylpiperazine N-2-ethanesulfonic acid; NEM, N-ethylmaleimide.

(1975) reported that certain amounts of calcium were required to induce coelomocyte clumping in *Asterias rubens*.

Factors other than calcium have also been implicated in sea star coelomocyte clumping *in vitro* (Boolootian and Giese, 1959; Jangoux and Vanden Bossche, 1975; Kanungo, 1982) and *in vivo* (Bang and Lemma, 1962; Bang, 1970; Reinisch and Bang, 1971; Reinische, 1974). These studies suggest that a factor released at the time the animal is wounded or challenged with foreign materials mediates coelomocyte clumping. A factor capable of inducing clumping in the coelomocytes of *A. forbesi* has been isolated from the coelomocytes of this sea star (Prendergast and Suzuki, 1970; Prendergast *et al.*, 1974). However, the existence of a clotting factor in the plasma (coelomic fluid free of coelomocytes = CF) of sea stars has not yet been demonstrated. The present study provides some experimental evidence for the existence of such a factor in the CF and examines the role of calcium and magnesium in agglutination of the coelomocytes of *A. forbesi*.

Materials and Methods

Animals

Asterias forbesi were purchased from the Marine Biological Laboratory, Woods Hole, Massachusetts. They were held in the laboratory at 12°C in 30-gallon aquaria with filtered, recirculating, continuously aerated sea water (salinity 30‰). No more than nine animals were kept in one aquarium, and the animals (wet weight 155–210 g) were used within ten days of their arrival in the laboratory.

Before the experiment the animals were screened under a low power dissecting microscope for surface wounds, and those without any visible wounds or abnormalities were used.

Collection of coelomic fluid

Two or three sea stars were removed from the holding tank, placed in a pail containing fresh sea water at room temperature (22°C), and held there for about 0.5 h.

The animal was blotted with a soft sponge and weighed. It was then held upright to allow the coelomic fluid to accumulate in the downward-hanging arms. When the arms were visibly swollen, the dermal papullae near the tip of the swollen arms were abraded with a razor blade. Coelomic fluid (1 ml) was allowed to drop into a 15-ml graduated centrifuge tube, coated inside with a thin layer of paraffin (melting point 60°C), which held 9 ml of calcium- and magnesium-free salt solution (CMFSS) containing 15 m*M* ethylenediamine tetracetic acid (EDTA). CMFSS was prepared by dissolving the following components in a liter of glass-distilled water: NaCl, 25.5 g; KCl, 0.8 g; Na_2SO_4, 3.0 g; glucose, 3.0 g; and Hepes (N-2-hydroxy-ethylpiperazine N-2-ethanesulfonic acid), 2.86 g. The pH of CMFSS and CMFSS-EDTA solutions was adjusted to 7.4 with NaOH. The solutions were filtered through presterilized 0.22-μm Millipore filters and stored in sterile containers until use.

Preparation of coelomocyte suspension

Soon after collection the coelomic fluid-CMFSS-EDTA solution was mixed by gentle pipetting several times through a Pasteur pipette. The resulting cell suspension was then centrifuged at $200 \times g$ for ten minutes. It was determined by light microscopy that the coelomocytes thus treated did not lyse or suffer visible damage.

Almost all cells in the suspension were separate and nonclumped. The supernatant was discarded and the cell pellet was resuspended in fresh CMFSS. Cell counts were made using a hemocytometer. Only nonclumped cells were counted, and the cell density was adjusted to about 10^6 cells/ml. Usually coelomocytes from several animals were pooled to run replicate experiments.

The test system

The experimental system we used to assess the sensitivity of coelomocytes to CF and to Ca^{2+} and/or Mg^{2+} is as follows. Cell suspension in CMFSS (10 ml) was placed in a 25-ml Erlenmeyer flask, the inside of which was coated with a thin layer of paraffin. This reaction flask was then placed in a shaker water bath at 20°C and agitated at 50–60 revolutions per minute. A count of nonclumped cells was made immediately after the cell suspension was placed in the flask. This count, taken at time zero, is referred to as the initial count. A test substance(s) (CF, $CaCl_2$, and/or $MgCl_2$) was then added (at various concentrations) to the suspension. Stock solutions (for CF see below) of the test salts were prepared in deionized water. Reagent grade chemicals were used in all experiments. The volume of a test substance(s) added to the reaction flask did not exceed 1% of the volume of cell suspension in the flask. The concentration of CF in the suspension is expressed as μl CF/ml CMFSS, whereas those of $CaCl_2$ and/or $MgCl_2$ are expressed in millimolar (mM) units of Ca^{2+} and Mg^{2+} assuming 100% dissociation of the salts in the test system. Control systems were prepared and incubated in exactly the same manner as the experimentals but contained appropriate volumes of deionized water in place of a test solution.

Nonclumped-cell counts were made at five-minute intervals for a total experimental period of 30 min. A significant decrease in nonclumped-cell number during an experiment was considered to be due to clumping of cells since other factors that could cause such a decrease in our *in vitro* system were eliminated (see below). Thus a concentration of a substance in the test system producing a significant decrease in nonclumped-cell count during the period of the experiment is referred to as "clumping concentration," and one that did not produce such a decrease is termed a "nonclumping concentration." Microscopic observations on samples taken from the reaction flasks were conducted along with the cell counts to determine if clumping of coelomocytes had actually occurred.

Tests for cell attachment and/or lysis

Since coelomocyte attachment to the vessel wall and/or lysis of these cells could cause a reduction in nonclumped-cell counts in our *in vitro* system, the following experiments were performed to determine if these possibilities existed in our test system.

(a.) A portion of cell suspension containing a clumping concentration of CF (10 μl/ml) or of Ca^{2+} and Mg^{2+} (0.23 and 0.12 mM, respectively) was placed on paraffinized slides and incubated in a humidified chamber at room temperature for 30 min. The suspension was drained off and the slide was inspected under a compound microscope for possible cell attachment. (b.) The cell suspension containing the above mentioned clumping concentration of CF or Ca^{2+} + Mg^{2+} was incubated for 30 min in a manner similar to other experimental systems described under the test system. After the incubation the cell suspension was centrifuged, and the pellet was resuspended in CMFSS containing 10 mM N-ethylmaleimide (NEM). A nonclumped-cell count was made to determine if the initial nonclumped-cell number was restored.

Preparation and assay of normal, dialyzed, and heated CF

Normal. Coelomic fluid was collected in a precooled, paraffinized centrifuge tube by abrading the animals as described above. It was then centrifuged at 200 × *g* for 10 min. The cell pellet was discarded, and the supernatant was filtered through a sterilized 0.22-μm millipore filter and stored at −20°C in sterile containers.

Experiments were performed with different concentrations of CF ranging from 5 to 10 μl/ml (at graded concentration intervals of CF) to establish a cutoff point between nonclumping and clumping concentrations of CF.

Dialyzed. Fifty ml of normal CF was dialyzed against 500 ml of CMFSS for 48 h at 4°C with constant stirring. CMFSS was changed five times during the 48-h period. At the end of this period, CF was sterilized by filtration and stored as discussed above.

The clumping effectiveness of dialyzed CF was tested by adding the CF at 10 μl/ml to the test system. This concentration was chosen because experiments with normal CF suggested this was a clumping concentration.

Heated. Normal CF was heated for 15 min at 100°C then cooled, sterilized by filtration, and stored as described above. This CF was also assayed at 10 μl/ml for its clumping effectiveness.

Experiments with Ca^{2+} and/or Mg^{2+}

Calcium and magnesium were assayed in absence of CF by adding various concentrations of these ions, independently of each other, to the test system. From these experiments nonclumping and clumping concentrations for Ca^{2+} and Mg^{2+} were established. Similarly, nonclumping and clumping concentrations of Ca^{2+} + Mg^{2+} were determined by assaying the two ions in combination in the test system.

"Reconstitution" experiments

These experiments were designed to test the clumping ability of dialyzed CF in the presence of Ca^{2+} and Mg^{2+}. Two series of experiments, were performed. (i.) initially dialyzed CF at 10 μl/ml (a clumping concentration for normal CF) was added to the test system. The system was then incubated for 30 min after which Ca^{2+} and Mg^{2+} were added to yield concentrations of 0.1 m*M* and 0.6 m*M*, respectively. These specific ion concentrations were used because normal CF when added to the test system at a concentration of 10 μl/ml yields 0.1 m*M* Ca^{2+} and 0.6 m*M* Mg^{2+} (see below for determination Ca^{2+} and Mg^{2+} in CF). Subsequent to the addition of the divalent cations in the entire system was further incubated for another 30-min period. Nonclumped-cell counts were made at 5- or 10-min intervals from the beginning of the experiment to the end of the second 30-min incubation period. (ii.) The sequence of addition of dialyzed CF and Ca^{2+} + Mg^{2+} to the test system was reversed. First Ca^{2+} and Mg^{2+} were added to obtain concentrations of 0.1 m*M* and 0.6 m*M*, respectively. The system was then incubated for 30 min following which dialyzed CF at a concentration of 10 μl/ml was added. Subsequently, the entire system was further incubated for another 30-min period. Cell counts were made during the entire 60-min incubation period as described in (i). In these two series of experiments the initial time of reconstitution refers to the time when the final ingredient(s) (dialyzed CF or the divalent cations) was (were) added to the test system.

Coelomocyte viability tests

After the experiment, viability of coelomocytes was determined by two separate methods: Trypan Blue exclusion method (Phillips, 1973), and visual observation of coelomocyte attachment and spreading on a glass surface. For the latter test a sample of postexperimental cells was placed on a clean glass slide and observed under a phase contrast microscope. Since only live coelomocytes can attach to a surface and spread by extending petaloid pseudopodia (formed by bladder coelomocytes which constitute over 90% of the total coelomocyte population [Kanungo, 1982]), those that attached and spread their pseudopodia were considered viable.

Ca^{2+}, Mg^{2+}, and osmolality determinations

Normal and dialyzed CF were analyzed on a flame photometer (Coleman Model 51, Coleman Instruments, Perkin-Elmer Corp., Maywood, IL) for their Ca^{2+} contents while their Mg^{2+} contents were determined using an atomic absorption spectrophotometer (Perkin-Elmer Model 560, Perkin-Elmer Corp., Maywood, IL.)

The osmolalities of various solutions were determined using an Advanced Digimatic Osmometer (Advanced Instruments, Inc., Needham Heights, MA). The instrument operates on freezing point depression principle and gives a readout in milliosmoles/kg.

Statistical analysis and calculations

Paired *t* tests were applied to compare cell counts at each time interval with that at zero time. Data were considered significant at the 95% confidence level.

In case where the number of nonclumped cells is expressed as % of the initial number, linear regression was used to determine the best fit lines (except where indicated otherwise).

The percent clumping was calculated by subtracting the mean cell count at a given time point from that at time zero (or at time 30 min in reconstitution experiments) and taking this difference as percent of the corresponding initial count.

Results

The assumption that a decrease in the number of single coelomocytes in our *in vitro* system was not due to attachement and/or lysis of cells but due to clumping must be justified, for if this assumption is not valid the results obtained by using this system would be meaningless.

The osmolalities of CMFSS and CF were 933 and 955 mOsmol/kg, respectively. They were, therefore, considered here as isoosmotic for all practical purposes. Thus, the coelomocytes could not have been osmotically stressed even though the coelomic fluid was diluted 10× with CMFSS during collection. Almost all cells remained viable during the experimental period as judged from post-experimental cell viability tests.

Cell attachment and/or lysis

Microscopic observations showed that the coelomocytes did not attach to paraffinized slides. However, the same cells, when placed on clean glass slides, adhered to the glass surface, extended petaloid pseudopodia, and subsequently underwent transformation from bladder to filiform type.

TABLE I

Changes in the number of nonclumped coelomocytes of the sea star Asterias forbesi *in the test system (cell suspension in CMFSS) and after exposure to clumping concentrations of CF or Ca^{2+} + Mg^{2+} for 30 min* in vitro *followed by exposure to 10 m*M *NEM.*

Time (min)	Number ($\bar{x} \pm SD \times 10^{-4}$/ml) of nonclumped coelomocytes		
	CMFSS	CF (10 μl/ml)	0.23 m*M* Ca^{2+} + 0.12 m*M* Mg^{2+}
	$n^{+} = 9$	$n = 6$	$n = 6$
0	104.00 ± 11.52	115.00 ± 8.17	125.83 ± 20.45
5	103.78 ± 18.53	—	—
10	101.00 ± 23.36	—	—
15	107.00 ± 22.46	—	—
20	100.89 ± 15.79	—	—
25	108.11 ± 21.38	—	—
30	104.56 ± 18.01	70.83 ± 8.86*	70.83 ± 10.57*
		↓ + NEM	↓ + NEM
45	—	109.17 ± 4.49	123.33 ± 32.30

* Significant at 95% confidence interval in paired comparison *t* tests between the indicated mean ($\bar{x}$) and that at time zero.

$^{+}$ n = number of experiments.

There was also no significant decrease in the number of nonclumped cells during the 30-min experimental period when the cells were suspended in CMFSS (Table I) or in diluted CMFSS as in control flasks. However, the possibility existed that a test substance in clumping concentration in the system might cause attachment of the coelomocytes to the vessel (cell lysis does not occur in CF or in Ca^{2+} and Mg^{2+}) and thus produce a decrease in nonclumped-cell count. This is discounted by the results given in Table I which show that after induction of clumping the initial number could be restored if the total cell population in the system was resuspended in NEM. Thus, the contention that the observed decrease in the number of coelomocytes in our test system was due to clumping and not due to attachment and/or lysis is fully justified.

Effects of CF

Normal CF. CF of *A. forbesi* contains on the average 10 m*M* Ca^{2+} and 60 m*M* Mg^{2+}. In our test system the number of nonclumped coelomocytes did not decrease significantly from that of the initial number when the cells were suspended in CMFSS containing CF at concentrations equal to or lower than 7 μl/ml (Table II). Increasing the CF concentration to 7.5 μl/ml or higher, however, resulted in significant decrease in nonclumped-cell number (Table II). The clumps produced by CF ranged from 2-cell to 5-cell aggregates.

The overall reduction in cell counts produced by CF at concentrations of 7.5 μl/ml and 10 μl/ml in a 30-min period were 22% and 33%, respectively (Fig. 1). Both reductions were significantly different from the corresponding counts at time zero (Table II). The greatest reduction in nonclumped cell counts was produced during the first five-min period when, on the average, a 19% clumping was observed. However, in the next 25-min period the two CF concentrations produced different patterns of clumping. Although a 3% increase was observed with the CF concen-

TABLE II

Effects of various concentrations and treatment of CF on the number of nonclumped coelomocytes of the sea star Asterias forbesi *at different time intervals under* in vitro *conditions.*

	Number ($\bar{x} \pm SD \times 10^{-4}$/ml) of nonclumped coelomocytes					
	Concentration of CF (μl/ml) in test flasks					
Time (min)	5 (n = 5)	7 (n = 5)	7.5 (n =7)	10 (n = 9)	10 (Dialysed) (n = 8)	10 (Heated) (n = 8)
0	104.00 ± 10.18	102.20 ± 12.06	102.43 ± 6.70	112.11 ± 9.22	118.13 ± 16.76	119.00 ± 22.96
5	107.20 ± 10.00	95.80 ± 13.36	84.43 ± 7.73*	88.11 ± 8.56*	119.38 ± 15.30	117.83 ± 23.44
10	101.40 ± 10.95	92.20 ± 20.54	—	89.89 ± 17.34*	113.75 ± 9.27	121.38 ± 40.73
15	—	86.20 ± 16.44	81.43 ± 7.73*	84.22 ± 14.33*	111.88 ± 18.86	117.00 ± 34.45
20	97.20 ± 16.36	91.00 ± 21.95	83.43 ± 8.52*	82.33 ± 18.22*	117.50 ± 19.69	110.50 ± 29.83
25	97.20 ± 12.25	88.60 ± 21.21	82.86 ± 7.38*	80.00 ± 14.51*	107.50 ± 19.36	107.13 ± 25.79
30	97.40 ± 8.16	97.80 ± 23.10	78.14 ± 10.25*	72.67 ± 9.52*	113.13 ± 10.88	111.25 ± 28.34

* Significant at 95% confidence interval.

tration of 7.5 μl/ml during this 25-min period, this was not significantly different from the 19% clumping produced in the initial period. (The regression line through these time points is, therefore, horizontal in Fig. 1.) On the other hand, a significant increase of 14% over the initial 19% was observed with a CF concentration of 10 μl/ml during the same 25-min period.

Dialyzed and heated CF. The osmolality of dialyzed CF was 933 mOsmol/kg, and such CF did not contain any detectable amount of Ca^{2+} or Mg^{2+}. The number of nonclumped coelomocytes did not decrease significantly with the addition of 10

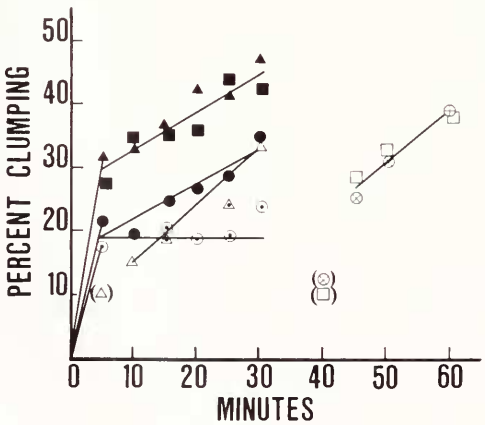

FIGURE 1. Effects of cell-free coelomic fluid (CF), Ca^{2+}, and/or Mg^{2+} on clumping of coelomocytes of *Asterias forbesi in vitro*. The number of replicate experiments (n) performed in each category is shown in Tables II and III except those of the "reconstitution" experiments. ⊙ and ● for CF concentrations of 7.5 μl/ml and 10 μl/ml, respectively. ▲ and ■ for 0.45 m*M* Ca^{2+} and 0.23 m*M* Ca^{2+} + 0.12 m*M* Mg^{2+}, respectively. △ for 0.75 m*M* Mg^{2+}. Reconstitution experiments (see text for details): □, initial addition of dialyzed CF (n = 10); ⊗, initial addition of Ca^{2+} + Mg^{2+} (n = 8). Symbols in ( ) represent the corresponding mean cell counts that are not significantly different from those at time zero, or, in "reconstitution" experiments, from those at 30 min.

μl/ml of dialyzed or heated CF to the system, even though this concentration was well above the minimal clumping concentration of normal CF (7.5 μl/ml) (Table II).

Effects of Ca^{2+} and/or Mg^{2+}

There was no significant decrease in nonclumped-cell numbers with addition of 0.23 m*M* Ca^{2+} or 0.50 m*M* Mg^{2+} to the test system (Table III). Similarly, no reduction in cell number was observed when 0.1 m*M* Ca^{2+} + 0.6 m*M* Mg^{2+} were added to the system. However, addition of 0.23 m*M* Ca^{2+} + 0.12 m*M* Mg^{2+}, or 0.45 m*M* Ca^{2+} produced a reduction of 45% in a 30-min period, whereas 0.75 m*M* Mg^{2+} reduced the cell number by about 32% from the initial during the same period (Fig. 1). In addition, the greatest amount of reduction was achieved during the first 5-min period when on the average a 30% decrease in nonclumped-cell counts was effected with the above concentrations of divalent cations (except with 0.75 m*M* Mg^{2+}). During the next 25-min period a reduction of about 15% in nonclumped-cell number was observed with 0.23 m*M* Ca^{2+} + 0.12 m*M* Mg^{2+} and with 0.45 m*M* Ca^{2+}. In Figure 1, one line is drawn through this 25-min period's time points because the calculated regression lines through the data points for the clumping concentrations of Ca^{2+} + Mg^{2+} and Ca^{2+} are very close to each other. It is therefore reasonable to conclude that coelomocyte clumping requires higher concentrations of Ca^{2+} or Mg^{2+} when these ions are used individually than when they are used together and that the divalent cations act synergistically in regard to clumping.

With 0.75 m*M* Mg^{2+} no significant decrease in nonclumped-cell count occurred during the initial 5-min period (Fig. 1). The large standard deviation associated with the mean suggests that there were excessive variations among the replicate counts taken at the end of the initial 5-min period (Table III).

Coelomocyte clumping pattern with "reconstituted" CF

The reconstitution experiments showed a decline in nonclumped cells by about 40% in 30 min after reconstitution. In Figure 1 the line through these time points is drawn by estimation since the regression lines through respective data points with

TABLE III

Effects of Ca^{2+}, Mg^{2+}, and Ca^{2+} + Mg^{2+} on the number of nonclumped coelomocytes of the sea star Asterias forbesi *at different time intervals under* in vitro *conditions.*

	Number ($\bar{x}$ ± SD × 10^{-4}/ml) of nonclumped coelomocytes					
	Conc. (m*M*) of Ca^{2+}		Conc. (m*M*) of Mg^{2+}		Conc. (m*M*) of Ca^{2+} + Mg^{2+}	
Time (min)	0.23 (n = 6)	0.45 (n = 6)	0.50 (n = 10)	0.75 (n = 5)	0.10 + 0.60 (n = 8)	0.23 + 0.12 (n = 11)
0	107.67 ± 21.57	113.29 ± 9.66	99.10 ± 10.79	112.20 ± 5.77	118.13 ± 14.35	122.73 ± 17.21
5	99.67 ± 25.73	78.43 ± 11.76*	96.30 ± 24.15	101.00 ± 22.00	113.75 ± 13.64	88.91 ± 10.89*
10	94.33 ± 21.88	76.43 ± 10.73*	88.80 ± 22.18	95.40 ± 11.52*	120.00 ± 9.35	80.00 ± 11.26*
15	104.67 ± 25.77	72.71 ± 12.59*	—	89.80 ± 13.86*	113.75 ± 15.56	79.82 ± 9.29*
20	104.33 ± 22.89	65.57 ± 10.07*	91.08 ± 25.29	—	112.50 ± 15.00	78.27 ± 15.90*
25	104.33 ± 20.14	66.86 ± 14.61*	89.50 ± 19.87	84.80 ± 5.27*	118.13 ± 15.80	68.64 ± 8.97*
30	118.33 ± 29.53	60.14 ± 15.85*	85.30 ± 19.64	74.20 ± 6.65*	113.13 ± 14.98	70.18 ± 11.15*

* Significant at 95% confidence interval.

these two sets of experiments are close to each other. The reconstitution experiments also reveal the following (ref. Fig. 1): (i.) The sequence of addition of dialyzed CF or the divalent cations to the system does not alter the extent of clumping after reconstitution as judged from the closeness of points in Figure 1. (ii.) The time course of clumping, for the 15-min period following reconstitution, with reconstituted CF is different from that with normal CF. In reconstitution experiments a lag period was evident when no significant reduction in nonclumped-cell numbers was observed until 15 min after reconstitution. (The two points corresponding to 40 min period in Figure 1 are not significantly different from zero clumping observed at the time of reconstitution.) (iii.) The extent of clumping at the end of 30 min after reconstitution (60 min from time 0) was 7% higher than that with normal CF at 10 μl/ml during a similar clumping period. This shows that the addition of the equivalent amount of divalent cations to dialyzed CF or *vice versa* restores the clumping effectiveness of dialyzed CF. Furthermore, it indicates a synergism between the divalent cations and the CF. (iv.) Since clumping could be achieved with the addition of 10 μl/ml of normal CF but not with the same concentration of dialzyed CF or equivalent concentrations of Ca^{2+} and Mg^{2+}, it is clear that CF-mediated coelomocyte clumping in our system is due to a factor(s) present in CF. (v.) From the conclusions stated in (iii) and (iv) above, it follows that the clumping factor(s) present in CF requires Ca^{2+} and Mg^{2+} to produce clumping of coelomocytes, and in the absence of these divalent cations the factor(s) is(are) ineffective as a clumping agent(s).

Discussion

The results indicate that coelomocytes clump when suspended in CMFSS containing clumping concentrations of CF, Ca^{2+}, and/or Mg^{2+}. The failure of the coelomocytes to clump when suspended in CMFSS, or in CMFSS containing CF below 7.5 μl/ml (Table II), 0.23 m*M* Ca^{2+}, 0.50 m*M* Mg^{2+}, or 0.1 m*M* Ca^{2+} + 0.6 m*M* Mg^{2+} (Table III), demonstrates that: (i) the clumping of these cells is dependent on the presence of Ca^{2+} and/or Mg^{2+} and a clumping factor(s) in the suspending medium; and (ii) a minimum concentration of Ca^{2+} and/or Mg^{2+} in the medium is necessary for clumping to occur. The necessary concentration of Ca^{2+} and/or Mg^{2+} for clumping is also dependent on whether or not the ions are used with CF, and whether they are used separately or in combination.

Since the agglutination of hemostatic cells in many animals, including mammalian platelets, is dependent on the presence of Ca^{2+} and Mg^{2+} in the medium (see review by Belamarich, 1976; Massini, 1977), it is not surprising to find that the coelomocytes of *A. forbesi* require these ions for clumping *in vitro*. However, the finding of Boolootian and Giese (1959) that clumping of the coelomocytes of 8 species of sea stars (which do not include *A. forbesi*) is independent of Ca^{2+} warrants critical examination in the light of the present results. The authors drew this conclusion because, in their system, sea star coelomocytes clumped in the presence of EDTA. In our collection system, which also contained EDTA, the coelomocytes remained separate and nonclumped. The species difference, while it could be a factor, is an unlikely explanation for this difference in results.

Thus the reason for coelomocyte clumping in the collection system of Boolootian and Giese must be sought in the technique used by the authors rather than in the species difference. They collected 0.9 ml of coelomic fluid in 0.1 ml of EDTA solution and used 2 m*M* and 13 m*M* EDTA at pH 7.6 in their final collection mixture. We used EDTA at a final concentration of 12.75 m*M* at pH 7.4. It is

unlikely that the pH difference would account for the diametrically opposite results obtained. However, it is possible that a certain amount of Ca^{2+} and/or Mg^{2+} was left unchelated in the system, and these free ions might have produced clumping. Thus, it becomes essential to discuss the kinetics of chelation of these divalent cations with EDTA in the system used by Boolootian and Giese.

The amount of EDTA present in the total 1 ml mixture of Boolootian and Giese was either 2×10^{-3} or 13×10^{-3} mmoles. The authors did not report the concentrations of Ca^{2+} and Mg^{2+} in the coelomic fluid of the sea stars they used. Thus, for purposes of present calculations, we have used the data reported by Binyon (1972) which show that the average concentrations of Ca^{2+} and Mg^{2+} in the coelomic fluid of four species of sea stars (*Astropecten* sp., *Solaster endica*, *Asterias vulgaris*, and *Marthaster glacialis*) are 10.8 m*M* and 46.4 m*M*, respectively. Accordingly, the respective amounts of these ions present in the coelomic fluid-EDTA mixture of Boolootian and Giese were 9.7×10^{-3} mmoles of Ca^{2+} and 41.8×10^{-3} mmoles of Mg^{2+}. Although the amounts of free Ca^{2+} and Mg^{2+} in the coelomic fluid would be less than the total amounts (because of association with other ions), it is reasonable to use the figures for the total amounts of these ions in calculations of their chelation with EDTA. The formation constants for Ca-EDTA and Mg-EDTA at pH 7 are 2.5×10^7 and 2.5×10^5, respectively (Kolthoff et al., 1969). Therefore, when both ion species are present in an equal mole ratio, Ca-EDTA is expected to be formed preferentially over Mg-EDTA in a ratio of 100:1. However, in the above coelomic fluid mixture the mole ratio of Ca^{2+}:Mg^{2+} = 1:4.3. This would produce Ca-EDTA and Mg-EDTA in an approximate ratio of 23:1. Since one mole of EDTA binds one mole of divalent cation, it follows that the maximum amount of divalent cation-EDTA complex that could be formed in the mixture is either 2×10^{-3} mmoles or 13×10^{-3} mmoles, depending on the concentration of EDTA used.

With a binding ratio of 23:1 and with 9.7×10^{-3} mmoles of Ca^{2+} present in the mixture, it can be easily calculated that 2×10^{-3} mmoles of EDTA could bind only 1.91×10^{-3} mmoles of Ca^{2+}. Therefore, 7.8×10^{-3} mmoles of Ca^{2+} and all the Mg^{2+} would be left uncomplexed in the mixture. However, with 13 m*M* EDTA, all Ca^{2+} present in the mixture would be chelated, while 38.5×10^{-3} mmoles of Mg^{2+} would be left uncomplexed. The concentration of this uncomplexed Mg^{2+} in the mixture is about 51× more than the clumping concentration (0.75 m*M*) reported here. Obviously, when the system contained 2 m*M* EDTA, uncomplexed Ca^{2+} had produced clumping. When it contained 13 m*M* EDTA, uncomplexed Mg^{2+} was probably responsible for clump induction. Further, our data show that Mg^{2+}, in the absence of Ca^{2+}, is not capable of maintaining clumps, provided the cell suspension in anticoagulant solution is stirred properly. Collecting coelomic fluid from the animals in a syringe, as was done by Boolootian and Giese, probably did not provide sufficient mixing of the fluid. This insufficient mixing together with uncomplexed Ca^{2+} or Mg^{2+} produced clumping in their collection system. Hence, their characterization that the clumping of sea star coelomocytes is independent of Ca^{2+} is unwarranted. The present findings and those of Jangoux and Vanden Bossche (1975) clearly demonstrate that the clumping of the sea star coelomocytes depends on the presence of Ca^{2+} and/or Mg^{2+} in the medium.

Clumping pattern with CF, Ca^{2+} and/or Mg^{2+}

The time course of clumping in the presence of CF at 10 μl/ml, 0.23 m*M* Ca^{2+} + 0.12 m*M* Mg^{2+}, or 0.45 m*M* Ca^{2+} is biphasic with an initial phase occurring during the first 5-min period and a second phase following (Fig. 1). This biphasic

mode of clumping, however, was not evident in two cases. The second phase clumping was absent with CF concentration of 7.5 μl/ml, while no initial phase could be discerned with 0.75 m*M* Mg^{2+} (Fig. 1).

Further, the data presented in Figure 1 reveal that the degree of clumping in the initial phase was variable and increased from 19% with CF to 33% with Ca^{2+} and Ca^{2+} + Mg^{2+} (except with 0.75 m*M* Mg^{2+}). Evidently not all coelomocytes that were potentially capable of clumping formed clumps with CF concentration of 7.5 or 10 μl/ml. It is interesting to note that the two clumping concentrations of CF produced identical clumping in the initial phase. Similarly, 0.45 m*M* Ca^{2+} and 0.23 m*M* Ca^{2+} + 0.12 m*M* Mg^{2+} also produced identical clumping in the initial phase (Fig. 1) even though they differed in their Ca^{2+} concentrations by a factor of 2. Since Mg^{2+} potentiates the clumping action of Ca^{2+}, a lower Ca^{2+} concentration in the presence of Mg^{2+} could produce clumping identical to that with a higher Ca^{2+} concentration in the absence of Mg^{2+}. Identical clumping rates observed with 0.23 m*M* Ca^{2+} + 0.12 m*M* Mg^{2+} and 0.45 m*M* Ca^{2+} might be coincidental and not necessarily indicative of maximal clumping for the initial phase. Variations in cell counts among replicate experiments which resulted in abolition of the initial phase in the case of 0.75 m*M* Mg^{2+} could have occurred if the individual cells forming clumps were not adhered to each other firmly. The "loose" clumps would dissociate easily and produce large variations in nonclumped-cell counts. This implies that with 0.75 m*M* Mg^{2+} it takes longer for the coelomocytes to develop "stickiness" and, therefore, more time is required to form "tight" clumps *in vitro*.

Sponge cells suspended in solutions containing EDTA suffer some damage and are inhibited from clumping, and this effect can be reversed by supplying proper amounts of Ca^{2+} and/or Mg^{2+} (Humphreys, 1963). EDTA inhibition of *Limulus* amoebocyte aggregation was reversed completely by adding 24 m*M* Mg^{2+} or *Limulus* "serum" at 1:20 dilution, but reversal was incomplete with 32 m*M* Ca^{2+} (Kenney *et al.*, 1972). Our results show that coelomocytes of *A. forbesi*, which have been inhibited from clumping with EDTA during collection and centrifugation, resume their clumping activity at a faster pace if immediately suspended in CMFSS containing clumping concentrations of CF, Ca^{2+} + Mg^{2+}, or Ca^{2+} than they do if immediately suspended in CMFSS containing a clumping concentration of Mg^{2+}.

During the second phase, although the overall extent of clumping increased in a linear fashion during a 25-min period, the rate of clumping was slower than that in the initial phase. Further, CF concentration of 10 μl/ml, 0.23 m*M* Ca^{2+} + 0.12 m*M* Mg^{2+}, and 0.45 m*M* Ca^{2+} produced a clumping rate of 6% per 10 min in the second phase, suggesting that the second phase clumping occurs independently of the initial phase. This conclusion is also supported by the results with CF at a concentration of 7.5 μl/ml which did not show any significant clumping in the second phase while producing a 19% clumping in the initial phase. In addition, a constant clumping rate during the second phase in contrast to variable rates in the initial phase suggests that the mechanism for coelomocyte clumping are different for each phase.

Clumping lag with reconstituted CF

The delay in initial clumping in reconstitution experiments could have been produced by the prolonged stay of the coelomocytes in dialyzed CF or nonclumping concentrations of Ca^{2+} + Mg^{2+}. Had EDTA produced any damage to the coelomocytes during collecting and centrifugation it could only have been accentuated by not returning the cells to a medium containing proper amounts of Ca^{2+}

and/or Mg^{2+}. Consequently, prolonged inhibition of clumping under these conditions would require longer recovery time and produce a lengthy lag period before clumping. This reasoning, while it explains the delay in initial clumping, also implies that clumping is brought about by the "stickiness" of the coelomocytes and that a nonclumping environment impairs the development of this "stickiness."

That the divalent cation chelators, EDTA and EGTA, may affect cell adhesion by removing materials from cell surfaces has been postulated for different cell types (Weiss, 1960; Curtis, 1973; Moscona, 1973). Nobel (1970) has also expressed similar views with regard to the effects of EDTA at pH 6.0 on the aggregation of the coelomocytes of the sea cucumber *Cucumaria frondosa.*

The coelomocyte-clumping factor in CF

The results presented here strongly suggest the existence of a coelomocyte clumping factor in CF which requires Ca^{2+} and Mg^{2+} to be effective. That the factor is nondialyzable and heat labile is also indicated by the results. It might be argued that dialyzing CF against CMFSS had removed not only Ca^{2+} and Mg^{2+} but also other constituents, such as trace elements and low molecular weight organic compounds, from CF. The removal of these other substances could affect clumping. Although the argument is reasonable, it is unlikely that these components exerted any effect on clumping of the coelomocytes. The restoration of clumping with reconstituted CF would not have been possible if components other than Ca^{2+} and Mg^{2+} had any appreciable effect on clumping.

Source and nature of clumping factor(s)

Two possible sources of the clumping factor(s) exist. The factor(s) could have been released (i) by the injured tissue, and/or (ii) from the coelomocytes during collection of the coelomic fluid and the preparation of the CF. Extracts of echinoderm tissues have been shown to cause coelomocyte clumping (Donnellon, 1938; Bookhout and Greenberg, 1940; Davidson, 1953; Boolootian and Giese, 1959). That an extract prepared from coelomocytes can mediate coelomocyte clumping in the sea star has also been demonstrated (Bang and Lemma, 1962; Bang, 1970). The latter observation has gained strength by the isolation and characterization of a clumping factor from the coelomocytes of *A. forbesi* (Prendergast and Suzuki, 1970; Prendergast and Liu, 1976). According to these authors, the factor is a basic protein with a molecular weight of approximately 38,000 daltons. The nondialyzability and the heat labile nature of the factor(s) reported here would also indicate that it (they) is (are) a protein(s). However, further experimental work is needed to determine the exact nature and the source of the clumping factor(s).

Mechanism of cellular clotting

Clotting of the coelomic fluid in echinoderms is achieved by the agglutination of the coelomocytes. Thus, an *in vitro* analysis of the mechanism of cellular aggregation (agglutination or clumping) is equated with the analysis of the mechanism of clotting.

Since Geddes (1880) first documented the cellular clotting in echinoderms many investigators have provided useful information on the subject (see review by Kanungo, 1982). However, except for the work of Boolootian and Giese (1959), none of the reports provide experimental studies on the clotting itself. The coelomocytes of all echinoderms form clumps *in vitro* (Endean, 1966; Johnson and Beeson, 1966;

Johnson, 1969; Bang, 1970; Chien *et al.*, 1970; Noble, 1970; Fontaine and Lambert, 1977; Bertheussen and Seljelid, 1978; Kaneshiro and Karp, 1980; Kanungo, 1982). In asteroid echinoderms, the predominant type of coelomocyte is the bladder amoebocyte, which takes part in clotting (Johnson and Beeson, 1966; Bang, 1970; Kanungo, 1982). Transformation of bladder to filiform amoebocytes, which was thought to be a prerequisite for cellular clotting in sea stars (Boolootian and Giese, 1958, 1959), has since been disputed by Johnson and Beeson (1966) and Kanungo (1982). Also, the contention of Boolootian and Giese (1959) that the cellular clotting in asteroid echinoderms does not require Ca^{2+} is no longer tenable in the light of the present findings and those of Jangoux and Vanden Bossche (1975).

Our results clearly demonstrate the requirement of Ca^{2+} and/or Mg^{2+} for clumping of the coelomocytes *in vitro*. Further, it is also shown that in the presence of CF (which contains the clumping factor) the requirement for these divalent cations for cellular clumping *in vitro* is less than it is without CF. Taken together these findings suggest that the clumping factor(s) alters the permeability of the cell membrane to divalent cations in a way which increases the influx of these ions. As a result, the intracellular concentrations of Ca^{2+} and Mg^{2+} increase to levels at which clumping becomes possible. In other words, the coelomocyte clumping depends on the intracellular rather than the extracellular concentrations of these ions. This is not unusual in light of the second messenger role played by Ca^{2+} in coordinating diverse cellular activities in many cell types (Rasmussen, 1970; Berridge, 1975), including mammalian platelets (Massini, 1977). The above hypothesis also predicts that in the absence of the clumping factor, a higher concentration of Ca^{2+} and/or Mg^{2+} in the medium would be required to establish a concentration gradient that would favor a greater influx of these ions which in turn would cause clumping. The present results substantiate this hypothesis because clumps could be formed with CF concentration at 7.5 μl/ml which contains 0.075 mM Ca^{2+} and 0.45 mM Mg^{2+}, but in the absence of CF clumping could not be effected even at concentrations of 0.1 mM Ca^{2+} + 0.6 mM Mg^{2+}.

Biphasic clumping and its implications

A two-stage coelomocyte clumping has been reported in the holothurian, *Cucumaria frondosa*, by Fontaine and Lambert (1977). The authors contended that the initial fast aggregating stage was brought about by the transitional cells which were present in the coelomic fluid before it was withdrawn from the animal, but the second slow phase was due to the transformation of the bladder amoebocytes to the transitional form which occurs at a slower pace *in vitro*.

It is, therefore, conceivable that the biphasic clumping reported here is due to two functional cell populations (a fast reacting population and a slow reacting one) which exist among the coelomocytes of *A. forbesi*.

The cells in the two groups probably differ in their permeability characteristics and their ability to establish surface properties for clumping. The fast reacting cells become "sticky" faster than the slow ones in the presence of a clumping stimulus. Whether these two populations of coelomocytes differ in their morphological characteristics is not known. Current investigations on intercellular adhesion implicate cell surface glycoproteins (Roseman, 1974; Oppenheimer, 1977, 1979) and lectins (Brown and Hunt, 1978; Rosen and Kaur, 1979) in generating sites for mutual adhesion of cells in a variety of cells systems. Similar studies using the coelomocytes of echinoderms would provide useful information for elucidating the mechanism of cellular clumping in these animals.

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